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29 SEP

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2. Patent application number *(The Patent Office will fill in this part)* — **9923060.9** — 29 SEP 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

CHIRON SpA
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53100 SIENA
ITALY

Patents ADP number *(if you know it)* — 7157811001

If the applicant is a corporate body, give the country/state of its incorporation — ITALY

4. Title of the invention — VACCINE

5. Name of your agent *(if you have one)* — **Carpmaels & Ransford**

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

43 Bloomsbury Square
London
WC1A 2RA

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Number of earlier application	Date of filing *(day / month / year)*

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Claim*(s)*	1
Abstract	1
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Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
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Carpmaels & Ransford

Date
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VACCINE

FIELD OF THE INVENTION

This application relates to mucosal DTP vaccines, especially intranasal vaccines.

BACKGROUND TO THE INVENTION

Bordetella pertussis is the causative agent of whooping cough. A highly effective inactivated whole cell vaccine has been available since the 1940s but concern over its safety, due to the presence of toxic cellular components, has limited its uptake [1]. Acellular pertussis vaccines (Pa) comprising a small number of defined *B.pertussis* antigens have therefore been produced, and have been approved for use in humans [2].

Pertussis vaccines are usually administered intramuscularly to children in the form of a trivalent DTP combination (diphtheria, tetanus, pertussis) on alum adjuvant. Intramuscular vaccination is not, however, the ideal route of administration. Mucosal vaccines (oral, intranasal *etc.*) are preferred for two reasons [3]. Firstly, they are easier to administer on a large scale, avoiding the need for specialised equipment and the problems associated with needles. Secondly, they stimulate mucosal immunity, mediated by secretory IgA. As most pathogens enter the body across mucous membranes, mucosal immunity is desirable.

Attempts to make acellular mucosal pertussis vaccines have been described [*eg.* 4, 5, 6, 7, 8,9], but the levels of protection reported were either not compared with conventional vaccine, or did not approach that observed the alum-adjuvanted antigens given parenterally.

There is therefore a need for an effective mucosal DTP combination vaccine.

DISCLOSURE OF THE INVENTION

The invention provides a mucosal DTPa vaccine comprising (a) a diphtheria antigen (D), a tetanus antigen (T), an acellular pertussis antigen (Pa), and (b) a detoxified form of either cholera toxin (CT) or *E.coli* heat labile toxin (LT).

The detoxified form of cholera toxin (CT) or *E.coli* heat labile toxin (LT) acts as a mucosal adjuvant [10]. Detoxification may be by chemical or, preferably, by genetic means. Suitable examples include LT having a serine residue at amino acid 63 ['LT-K63' – ref. 11], and LT having an arginine residue at amino acid 72 ['LT-R72' – ref. 12]. CT and LT are homologous and are typically interchangeable.

The mucosal vaccine of the invention is preferably an intranasal vaccine. In such embodiment, it is preferably adapted for intranasal administration, such as by nasal spray, nasal drops, gel or powder [13].

The acellular pertussis antigen preferably comprises pertussis holotoxin (PT) and filamentous haemagglutinin (FHA). It may further comprise pertactin and, optionally, agglutinogens 2 and 3 [14, 15].

PT is a toxic protein and, when present in the pertussis antigen, it is preferably detoxified. Detoxification may be by chemical and/or genetic means. A preferred detoxified mutant is the 9K/129G double mutant [2], referred to herein as 'rPT'.

The diphtheria antigen (D) is preferably a diphtheria toxoid, more preferably the CRM197 mutant [10]. The tetanus antigen (T) is preferably a tetanus toxoid [16].

Non-DTP antigens, preferably ones that do not diminish the immune response against existing components, may also be included [*eg.* ref. 17, which includes a HBV antigen, and ref. 18].

The invention also provides a method of raising an immune response in a patient, comprising administering to a patient a vaccine according to the invention. The immune response is preferably protective against whooping cough, diphtheria and tetanus. The patient is preferably a child.

The method may raise a booster response, in a patient that has already been primed against *B.pertussis.* The primer vaccination may have been by a mucosal or parenteral route.

The invention also provides the use of a detoxified mutant of cholera toxin (CT) or *E.coli* heat labile toxin (LT) in the manufacture of an intranasal medicament for vaccinating a patient against whooping cough, diphtheria and tetanus, or for boosting an primer immune response previously raised against *B.pertussis*.

It will be appreciated that references in the above text to particular proteins (*eg.* pertactin, PT, *etc.*) encompass their allelic variants and functional mutants. They also encompass proteins having significant sequence identity to the wild-type proteins. The degree of identity is preferably greater than 50% (*eg.* 65%, 80%, 90%, or more) calculated using, for instance, the Smith-Waterman homology search algorithm as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with parameters *gap open penalty=12* and *gap extension penalty=1*. Immunogenic fragments of these proteins may also be used, as may longer proteins incorporating the proteins, variants or fragments (*eg.* fusion proteins). In all cases, however, the protein (whether wild-type, variant, mutant, fragment or fusion) will substantially retain the wild-type immunogenicity.

The proteins can, of course, be prepared by various means (*eg.* recombinant expression, purification from cell culture, chemical synthesis *etc.*) and in various forms (*eg.* native, fusions *etc.*). They are preferably prepared in substantially pure or isolated form (*ie.* substantially free from other bacterial or host cell proteins with which they are normally associated in nature)

The vaccines of the invention may comprise nucleic acid for 'genetic immunisation' [*eg.* 19]. The nucleic acid will encode a protein component of the vaccine and may replace individual protein components, or may supplement them. As an example, the vaccine may comprise DNA that encodes a tetanus toxin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows Th1 and Th2 responses to an intranasal Pa vaccine adjuvanted with LT-K63. **Figure 2** shows similar data for LT-R72 adjuvant. **Figure 3** shows antibody responses for the vaccines used in Figures 1 & 2. **Figure 4** shows the kinetics of *B.pertussis* clearance after immunisation with the vaccines used in Figures 1 & 2.

Figure 5 shows the clearance kinetics after intranasal immunisation with a combined DTPa vaccine + LT-K63, and **Figure 6** shows the TT- and DT-specific cellular and humoral responses generated by the same vaccine.

Figure 7 shows T-cell proliferation against the D, T and Pa components of DTPa vaccines administered using different prime and boost regimens. The T-cell cytokine responses against the Pa component (**Figure 8**), the D component (**Figure 9**) and the T component (**Figure 10**) are also shown. **Figure 11** shows the IgG responses against the five defined antigens in the DTPa mixture, and **Figure 12** shows the IgA responses. **Figure 13** shows the functionally important anti-DT neutralising antibodies, and **Figure 14** shows clearance kinetics for various prime and boost regimens tested.

MODES FOR CARRYING OUT THE INVENTION

Background materials & methods

Mice used in the following examples were female BALB/c mice, 6-8 weeks old, from Harlan UK and were housed according to the regulations of the Irish Department of Health.

Th1/Th2 responses Mice were immunised at 0 and 4 weeks. At 6 weeks, spleen, superior cervical lymph nodes and posterior mediastinal (thoracic) lymph nodes were removed and immune responses were evaluated. Spleen cells from individual mice or pooled lymph node cells (2×10^6 cells/ml) from naïve or immunised mice were cultured in triplicate in 8% FCS supplemented RPMI at 37°C with heat-killed (80°C for 30 minutes) *B.pertussis* bacteria (10^6,10^7 cells/ml), heat inactivated rPT (1-5μg/ml), or FHA (1-5μg/ml). Phorbal myristate acetate (PMA) + anti-mouse CD3 was used as a positive control; medium only was used as a negative control. Supernatants were removed after 72 hours and the concentration of IFN-γ (indicative of Th1 response) and IL-5 (indicative of Th2 response) was determined by immunoassay as described in reference 20. T-cell proliferation was assessed after 4 days of culture by ^{3}H-thymidine uptake, also as described in reference 20.

Antibody assays Levels of antigen-specific IgG in the serum of control and immunised mice were determined by ELISA. Purified antigens (FHA, PT, TT and DT; 1.0μg/ml) were used to coat the ELISA plates. The plates were blocked with milk protein, then serially diluted serum samples were added, the bound antibody was detected by anti-mouse IgG (Fc-specific) alkaline-phosphatase conjugate (Sigma). Antigen-specific IgA in lungs was detected by ELISA. Lungs were homogenised in 8% FCS supplemented RPMI containing PMSF protease inhibitor. ELISA plates were coated with antigen as for the IgG assay and serially diluted lung homogenate was added. Bound antibody was detected with sheep anti-mouse IgA, followed by donkey-anti-sheep IgG alkaline phosphatase conjugate (Sigma).

1) LT mutants are intranasal adjuvants for Pa

Two Pa vaccines were prepared. The antigen component in each vaccine was FHA (2.5μg/dose) + rPT (5.0μg/dose), with antigens prepared as described in reference 21.

The first vaccine (Figure 1) was adjuvanted with LT-K63 (10μg/dose), whereas the second vaccine (Figure 2) was adjuvanted with LT-R72 (1μg /dose). A control vaccine consisted of FHA + rPT only. The adjuvants were prepared as described in references 22 and 23.

Mice were lightly anaesthetised with halothane, and were immunised at 0 and 4 weeks with vaccine resuspended in 50μl, by application to the external nares with a micropipette. T-cell responses to killed *B.pertussis*, heat-inactivated PT and FHA were measured in spleen and thoracic and cervical lymph nodes at 6 weeks (Figures 1 & 2).

Strong T-cell proliferation and cytokine production was detected for the adjuvanted Pa vaccines. In contrast, spleens and local lymph nodes from mice intranasally immunised with the control failed to generate significant *B.pertussis*-specific T-cell responses. Positive responses to the polyclonal stimulus (PMA + anti-CD3) confirms that these T-cells were capable of responding *in vitro*.

Figure 3 shows that the mutant LT adjuvants also enhanced local and systemic antibody production following intranasal delivery of Pa. Immunisation with the control generated weak and inconsistent anti-PT and anti-FHA serum IgG and lung IgA responses. In contrast, formulation of the same antigens with LT-R72 or LT-K63 resulted in consistently strong serum IgG and lung IgA specific for PT and FHA.

The presence of the LT mutants thus resulted in better T-cell and antibody responses. They can enhance the protective efficacy of a nasally delivered Pa, and are therefore effective intranasal adjuvants for acellular vaccines.

2) Protection against pertussis infection

Vaccine efficacy in human clinical trials has been correlated with the protection of immunised mice in the respiratory challenge model described in reference 20. This model was therefore used to assess the protective efficacy of intranasally delivered Pa formulated with the LT mucosal adjuvants, in order to predict human efficacy.

B.pertussis W28 phase I was grown under agitation conditions at 37°C in Stainer-Scholte liquid medium. Bacteria from a 48 hour culture were resuspended at a concentration of approximately 2×10^{10}cells/ml in physiological saline containing 1% casein. The challenge inoculum was administered to mice over a period of 15 minutes by means of a nebuliser, followed by rest in the chamber for a further 15 minutes. Groups of 4 mice were sacrificed at 0, 3, 7, 10 and 14 days, and the number of viable *B.pertussis* in the lungs were assessed. Lungs were removed aseptically from the infected mice and homogenised in 1ml sterile physiological saline with 1% casein on ice. Aliquots of 100μl undiluted or serially diluted homogenate from individual lungs were spotted in triplicate onto Bordet-Genou agar plates, and the number of colonies was assessed after 5 days incubation. Results are reported as the mean viable *B.pertussis* for individual lungs from four mice per time point per experimental group.

As shown in Figure 4, the adjuvanted Pa formulations provided levels of protect significantly greater than those achieved with soluble antigens alone. The LT-K63 adjuvant generated marginally better protection than LT-R72. Nasal delivery of Pa with LT-R72 in 25μl (no anaesthetic) gave marginally better protection than the same vaccine in 50μl (with anaesthetic). Neither of these two differences was significant.

The protection levels shown in Figure 4 exceed those previously observed with a conventional parenterally delivered two component Pa (25μg FHA + 25μg chemically-detoxified PT on alum [14]). Extrapolation of the correlation curve shows a better potency index, suggesting superior clinical efficacy in humans.

3) DTPa efficacy using LT-K63

Pertussis vaccines are usually administered intramuscularly to children in the form of a trivalent DTP combination on alum adjuvant. To assess the efficacy of intranasal vaccination, a DTPa vaccine was therefore adjuvanted with alum (300μg/dose, 300μl volume) for intramuscular administration, for comparison with the LT-K63-adjuvanted intranasal vaccine (10μg adjuvant/dose, 40μl volume). The Pa component of the vaccine included 5μg rPT, 2.5μg FHA and 2.5μg pertactin. The T component was 10μg tetanus toxoid, and the D component was 10μg CRM197.

The intranasal vaccine enhanced local and systemic Th1 and Th2 responses, serum IgG response, and lung IgA responses. The serum IgG levels for the three pertussis antigens were equivalent to those following intramuscular immunisation with the alum-adjuvanted vaccine, but the mucosal immunisation advantageously enhanced local IgA responses. Protection against *B.pertussis* challenge did not differ significantly for the two vaccines (Figure 5) and, whilst the clearance kinetics varied, they were equally protective.

The intranasal vaccine also induced cellular and humoral immune responses to tetanus and diphtheria (Figure 6). The anti-TT and anti-DT serum IgG levels were equivalent to the alum-based vaccine, but IgA levels were advantageously enhanced. Furthermore, TT- and DT-specific T-cell proliferation, IL-5 production and IFN-γ production were detected in spleen cells from mice immunised by both routes.

This is the first disclosure of a mucosally-delivered combined DTPa formulation that is capable of generating a level of protection against *B.pertussis* infection equivalent to that observed with the same antigens adsorbed on alum and administered parenterally.

4) Intramuscular priming and intranasal booster

The DTPa vaccine was also used in a prime-boost experiment.

Two groups of 22 mice were immunised intramuscularly at 0 and 4 weeks with either DTPa on alum, or PBS (control). A further group of 22 mice was immunised intranasally at 0 and 4 weeks with the LT-K63-adjuvanted vaccine. Two further groups of 22 mice were immunised with the intramuscular alum formulation at week 0, and the intranasal LT-K63-adjuvanted formulation at week 4.

Five mice from each group were sacrificed at week 6, and serum, lungs and spleen cells were measured for immune responses. The remaining mice were subjected to the infection model. One mouse from each group on day 0 and four mice from each group on days 3, 7, 10 and 14 were sacrificed, and their CFU-counts were measured from their lungs.

T-cell proliferation (Figure 7) was weak for spleen cells for all groups when stimulated with the pertussis antigens *in vitro*. The cells did, however, proliferate in response to the polyclonal activators (PMA+CD3). Proliferation responses to tetanus toxoid *in vitro* were significantly stronger in intranasally-boosted mice (after intramuscular priming) when LT-K63 was used as adjuvant. The strongest *in vitro* proliferation against the diphtheria component was seen in the mice immunised intranasally twice.

Cytokine responses to pertussis antigens (Figure 8) showed both IL-5 and IFN-γ production in all groups, indicating priming of both Th1 and Th2 populations *in vivo*. IL-4 production was limited to groups immunised in the same way both times. Priming and boosting with the intranasal LT-K63 formulation seems to give a stronger Th2 response (higher IL-4 and IL-5) than the groups primed intramuscularly.

Cytokine responses against the diphtheria antigen (Figure 9) were restricted to IL-4 and IL-5, with little or no IFN-γ detected for any group. Intranasal boosting with DTPa thus results in the priming of Th2 cells *in vivo*. The strongest Th2 response was generated from the mice immunised intranasally twice with the LT-K63 adjuvant. In contrast, two intramuscular injections gave no detectable IL-4 or IL-5 responses in the spleen, nor nay IFN-γ.

Cytokine responses against the tetanus antigen (Figure 10) showed the production of IL-4, IL-5 and low levels of IFN-γ in all mice, indicating a mixed Th1/Th2 response. IL-4 and IL-5 levels were, however, significantly higher in groups boosted intranasally with the LT-K63 adjuvant, compared with the non-adjuvanted intranasal booster or the intramuscular booster.

IgG responses against TT, DT and PTN (Figure 11) showed no significant differences in titr between the various groups. Anti-PT and anti-FHA titres were slightly higher in groups primed and boosted with DTPa intramuscularly than in groups boosted intranasally (with or without LT-K63 adjuvant). Anti-FHA IgG were not detected, although this is not in agreement with the results presented above.

IgA levels (Figure 12) showed that intramuscular priming and intranasal boosting using the LT-K63 adjuvant generated similar titres for most antigens to intranasal priming and boosting, although anti-PT levels were significantly lower. Intranasal boosting without the LT-K63 adjuvant generated lower IgA levels, particularly for DHA and PTN.

Analysis of functionally important anti-DT neutralising antibodies in murine sera (Figure 13) demonstrated that intramuscular priming and intranasal boosting using LT-K63 resulted in the highest levels.

The protection model showed that similar levels of protection was obtained in the dual-intranasal and dual-intramuscular immunisations. Whilst the kinetics of the clearance curves (Figure 14) vary, *B.pertussis* was effectively cleared in both cases, with CFU counts below 1 ($\log_{10}$) 14 days after challenge. The level of protection was lower where priming was intramuscular and boosting was intranasal, but boosting in the absence of LT-K63 appeared to be more effective than boosting in its presence.

Most adults today have received an intramuscular pertussis vaccination. This is represented by the intramuscular priming in this example. The example shows that intranasal boosting with LT-K63 adjuvant is an effective method of vaccination.

This example also shows that LT-K63 is a very effective adjuvant for the delivery of 1X CRM197. Intranasal enhancement against this antigen has been reported using chitosan, although this required three immunisations for modest IgA and T-cell responses. LT-K63 was able to induce strong IgG, IgA, IL-4 and IL-5 responses after two intranasal immunisations. Similar levels of anti-DT neutralising antibodies were also generated as with chitosan.

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

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CLAIMS

1. A mucosal DTPa vaccine comprising:
   (a) a diphtheria antigen, a tetanus antigen, and an acellular pertussis antigen; and
   (b) a detoxified form of either cholera toxin or *E.coli* heat labile toxin.

2. The DTPa vaccine of claim 1, wherein component (b) is LT-K63 or LT-R72.

3. The DTPa vaccine of claim 1 or claim 2, adapted for intranasal administration.

4. The DTPa vaccine of any preceding claim, wherein the acellular pertussis antigen comprises detoxified pertussis holotoxin and filamentous haemagglutinin and, optionally, pertactin.

5. The DTPa vaccine of claim 4, wherein the detoxified pertussis holotoxin is a 9K/129G double mutant.

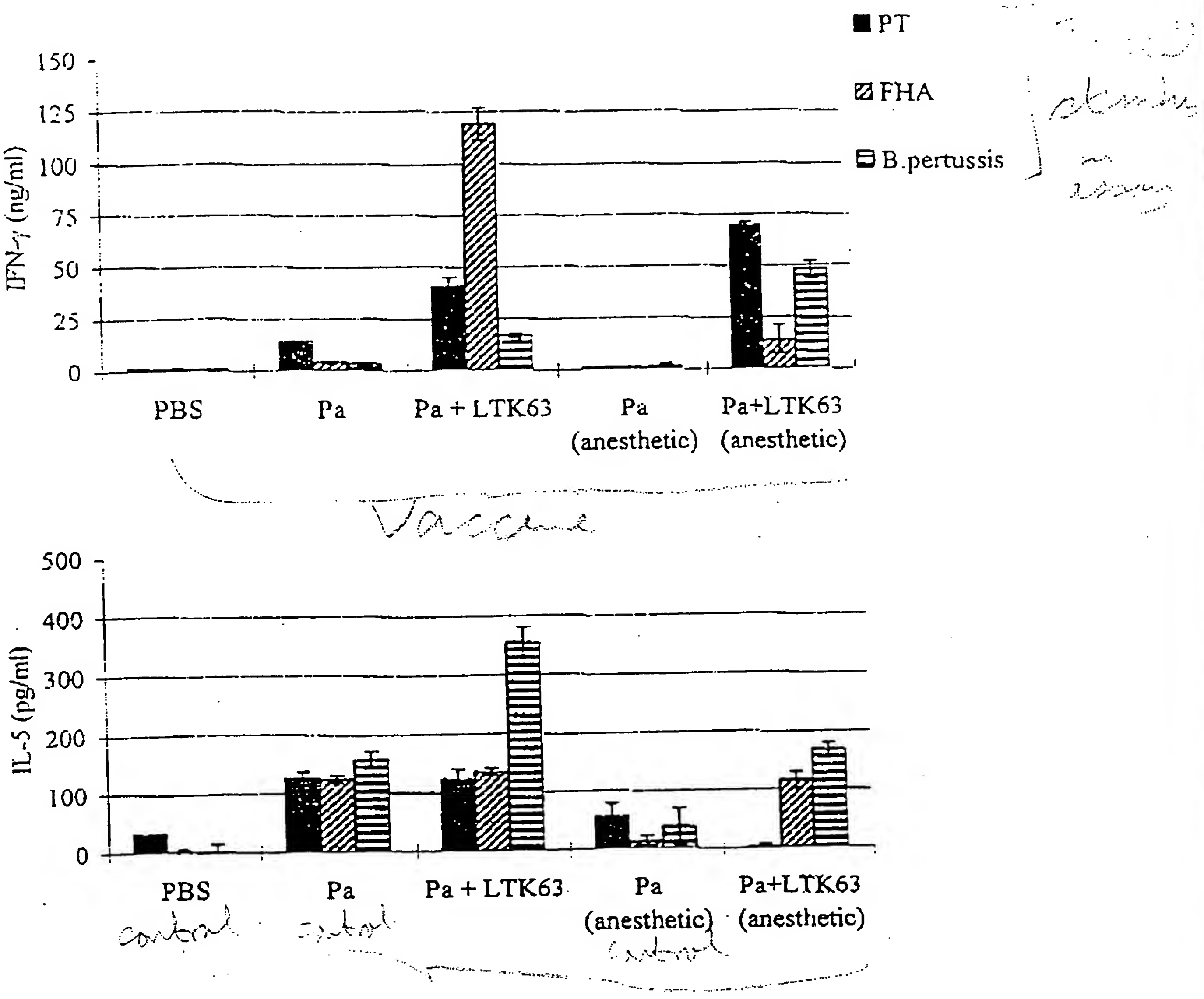
6. The DTPa vaccine of any preceding claim, wherein the diphtheria antigen is the CRM197 mutant and the tetanus antigen is a tetanus toxoid.

7. A method of raising an immune response in a patient, comprising administering to a patient a vaccine according to any preceding claim.

8. The use of a detoxified mutant of cholera toxin or *E.coli* heat labile toxin in the manufacture of an intranasal medicament for vaccinating a patient against whooping cough, diphtheria and tetanus.

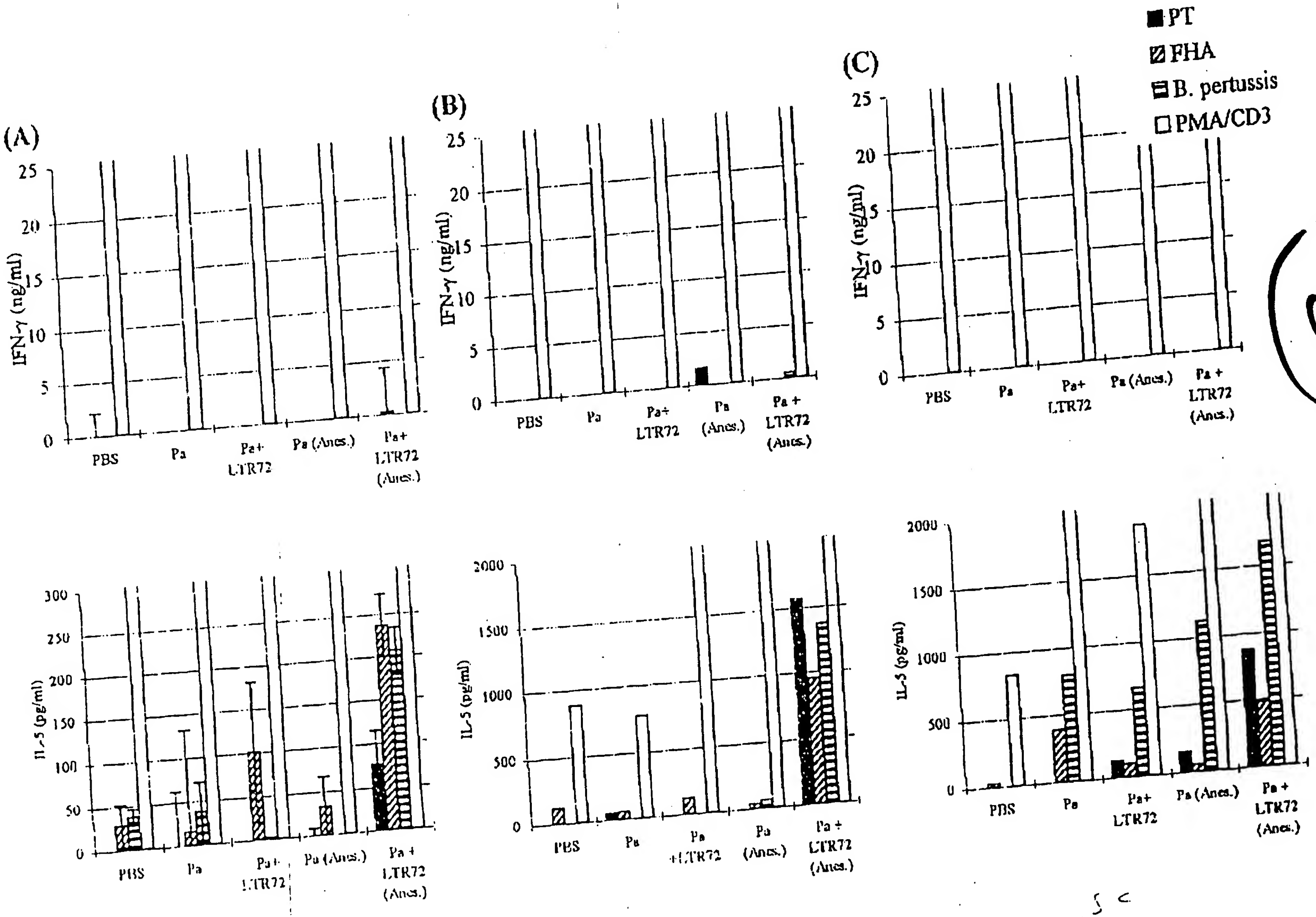
ABSTRACT

Mucosal DTPa vaccines, especially intranasal vaccines, comprising (a) a diphtheria antigen, a tetanus antigen and an acellular pertussis antigen, and (b) a detoxified mutant of cholera toxin (CT) or *E.coli* heat labile toxin (LT). Component (b) acts as a mucosal adjuvant. The acellular pertussis antigen preferably comprises pertussis holotoxin (PT) and filamentous haemagglutinin (FHA) and, optionally, pertactin.


■ PT
▨ FHA
▤ B.pertussis
stimulus in assay
IFN-γ (ng/ml)
150
125
100
75
50
25
0
PBS
Pa
Pa + LTK63
Pa (anesthetic)
Pa+LTK63 (anesthetic)
Vaccine
IL-5 (pg/ml)
500
400
300
200
100
0
PBS
control
Pa
control
Pa + LTK63
Pa (anesthetic)
control
Pa+LTK63 (anesthetic)

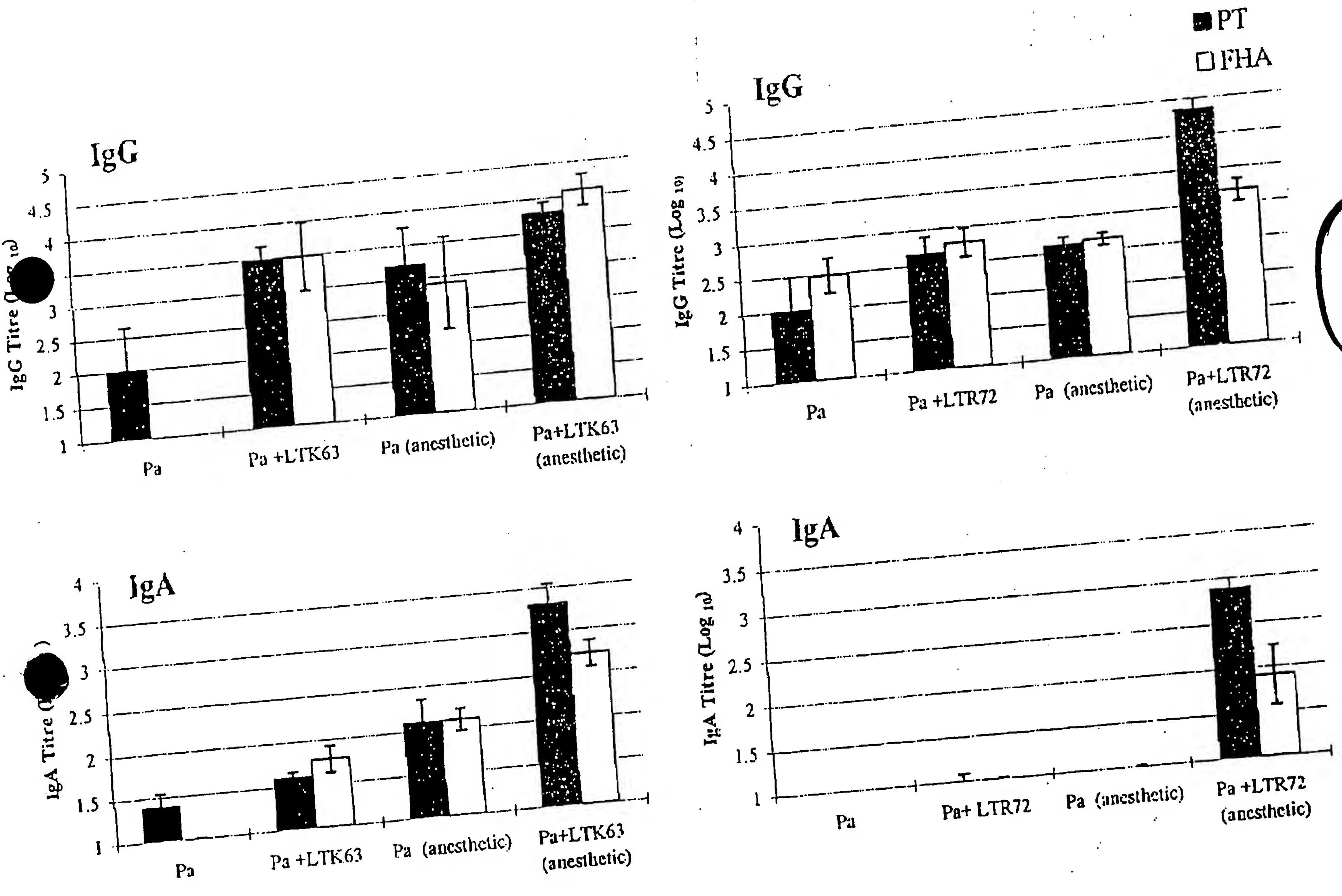
①

Fig. 1


(A)
(B)
(C)
IFN-γ (ng/ml)
0
5
10
15
20
25
IL-5 (pg/ml)
0
50
100
150
200
250
300
500
1000
1500
2000
PBS
Pa
Pa+ LTR72
Pa (Anes.)
Pa + LTR72 (Anes.)
■ PT
▨ FHA
▤ B. pertussis
☐ PMA/CD3

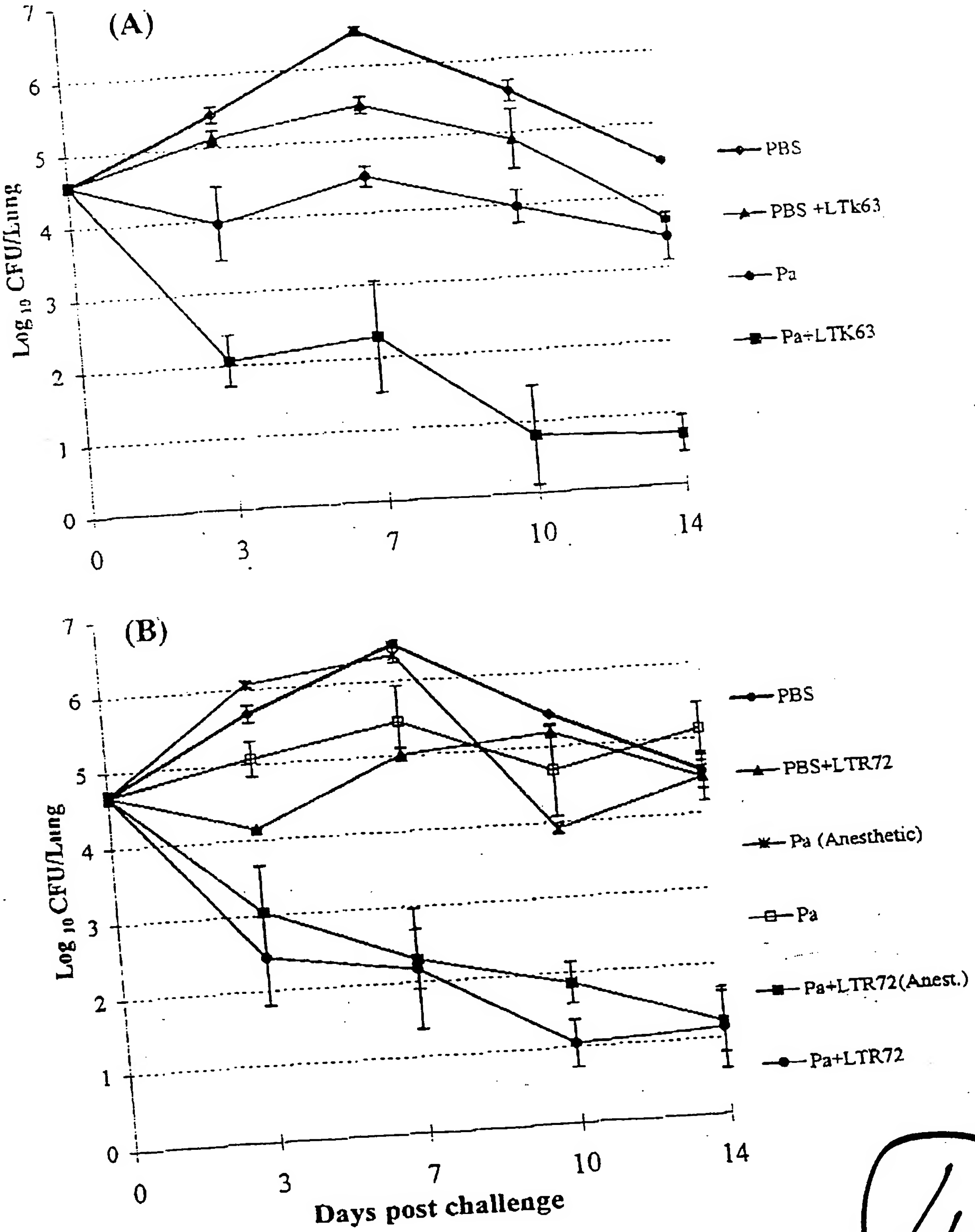
2

Fig. 2


■ PT
□ FHA
IgG
IgG Titre (Log 10)
5
4.5
4
3.5
3
2.5
2
1.5
1
Pa
Pa +LTK63
Pa (anesthetic)
Pa+LTK63 (anesthetic)
IgG
IgG Titre (Log 10)
Pa
Pa +LTR72
Pa (anesthetic)
Pa+LTR72 (anesthetic)
IgA
IgA Titre
4
3.5
3
2.5
2
1.5
1
Pa
Pa +LTK63
Pa (anesthetic)
Pa+LTK63 (anesthetic)
IgA
IgA Titre (Log 10)
Pa
Pa+ LTR72
Pa (anesthetic)
Pa +LTR72 (anesthetic)

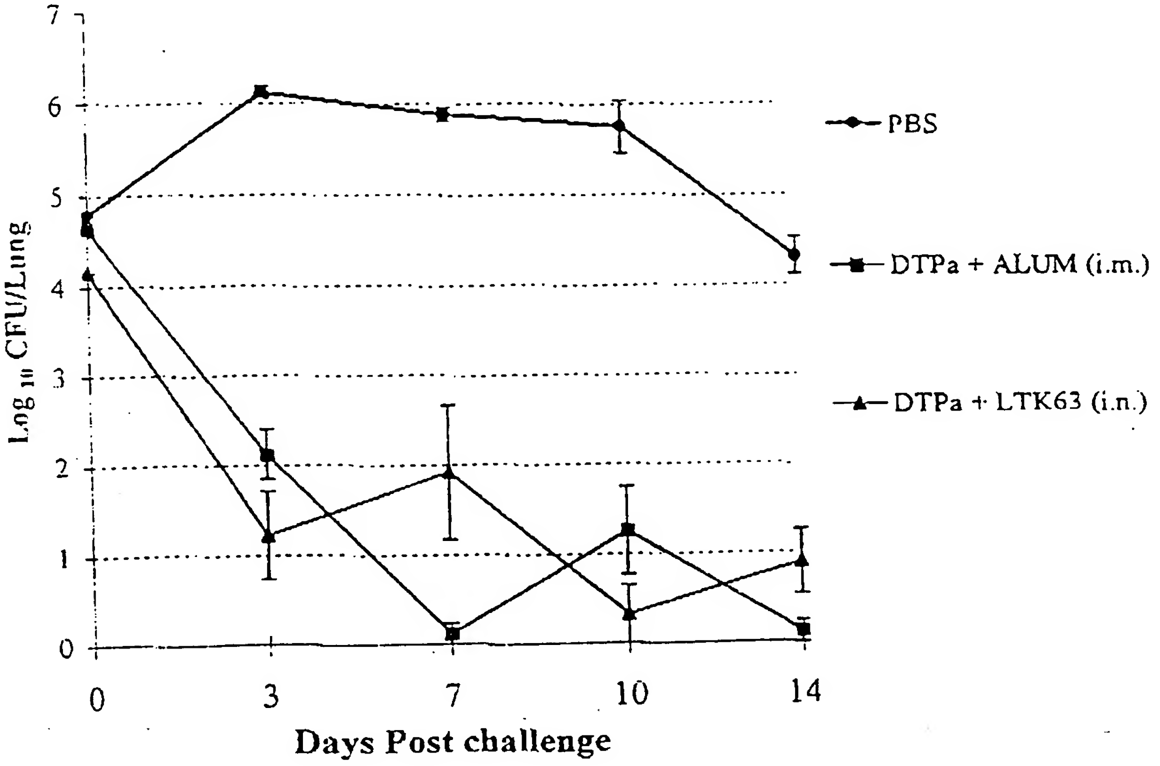
3

Fig. 3


(A)
Log 10 CFU/Lung
7
6
5
4
3
2
1
0
0
3
7
10
14
PBS
PBS +LTk63
Pa
Pa+LTK63
(B)
Log 10 CFU/Lung
7
6
5
4
3
2
1
0
0
3
7
10
14
Days post challenge
PBS
PBS+LTR72
Pa (Anesthetic)
Pa
Pa+LTR72(Anest.)
Pa+LTR72

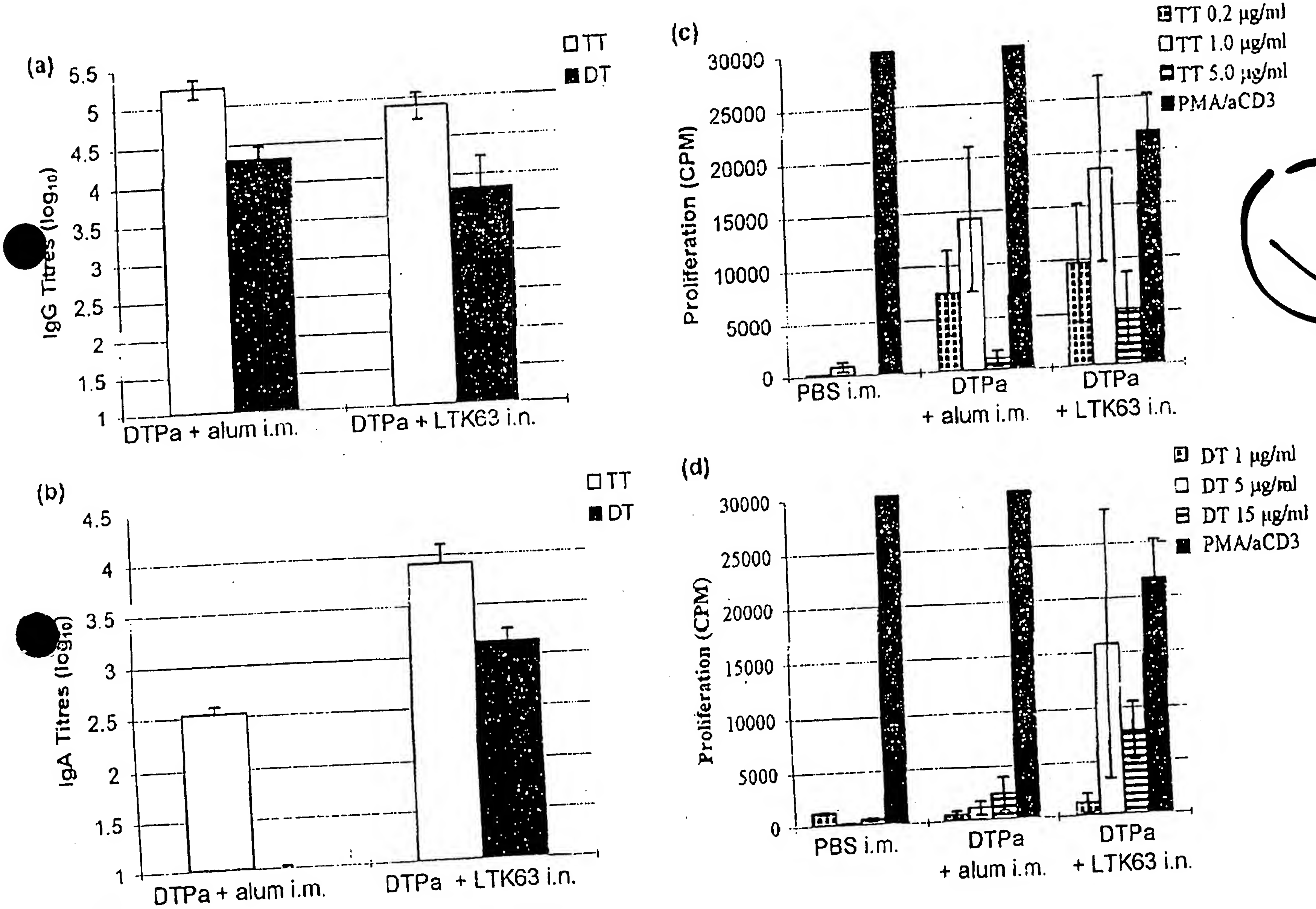
④

Fig. 5


Log 10 CFU/Lung
7
6
5
4
3
2
1
0
0
3
7
10
14
Days Post challenge
PBS
DTPa + ALUM (i.m.)
DTPa + LTK63 (i.n.)

(5)

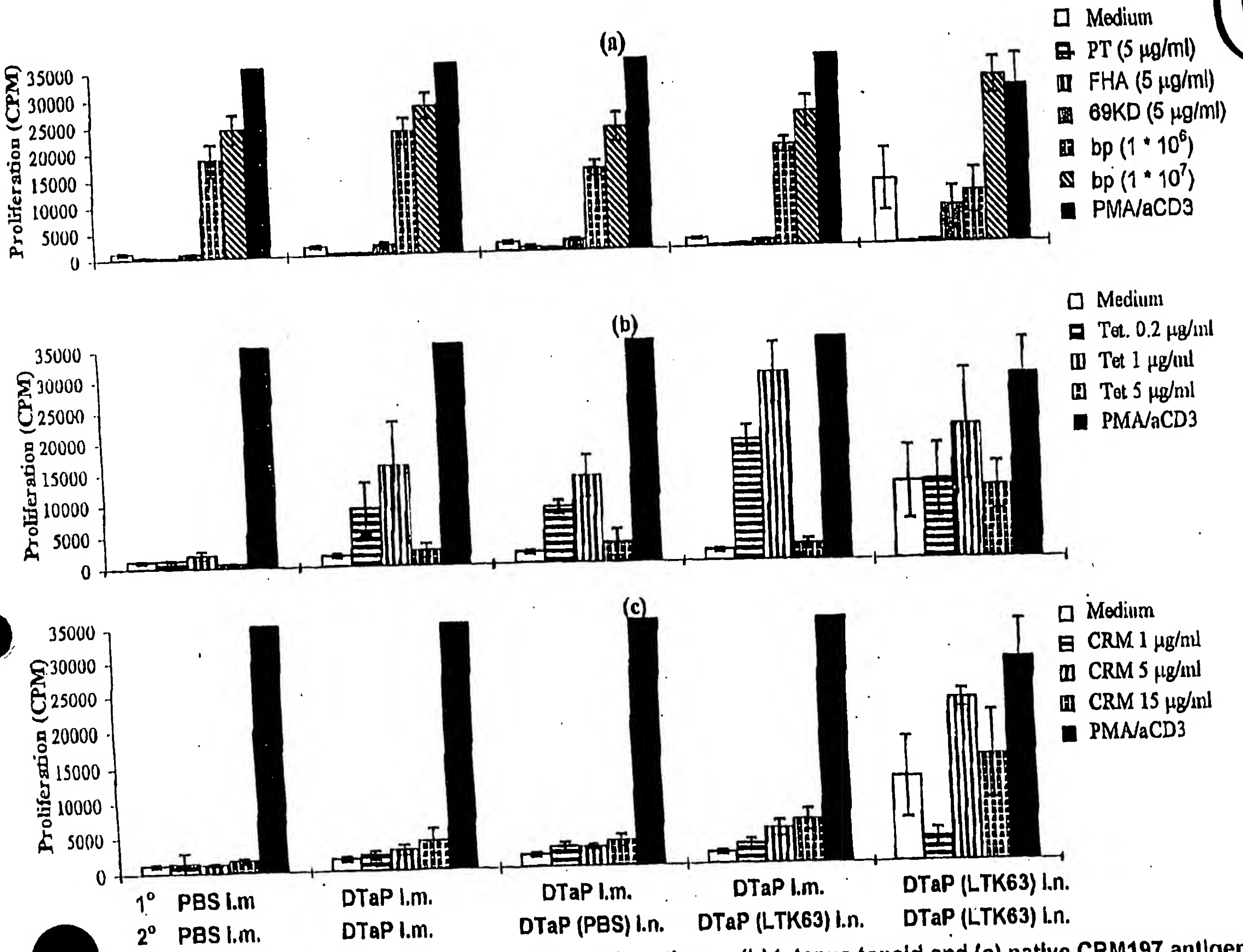
Fig. 6


(a)
IgG Titres ($\log_{10}$)
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
DTPa + alum i.m.
DTPa + LTK63 i.n.
□ TT
■ DT
(b)
IgA Titres ($\log_{10}$)
4.5
4
3.5
3
2.5
2
1.5
1
DTPa + alum i.m.
DTPa + LTK63 i.n.
□ TT
■ DT
(c)
Proliferation (CPM)
30000
25000
20000
15000
10000
5000
0
PBS i.m.
DTPa + alum i.m.
DTPa + LTK63 i.n.
▦ TT 0.2 µg/ml
□ TT 1.0 µg/ml
▤ TT 5.0 µg/ml
■ PMA/aCD3
(d)
Proliferation (CPM)
30000
25000
20000
15000
10000
5000
0
PBS i.m.
DTPa + alum i.m.
DTPa + LTK63 i.n.
▦ DT 1 µg/ml
□ DT 5 µg/ml
▤ DT 15 µg/ml
■ PMA/aCD3

6

277 19/5/77. (F)

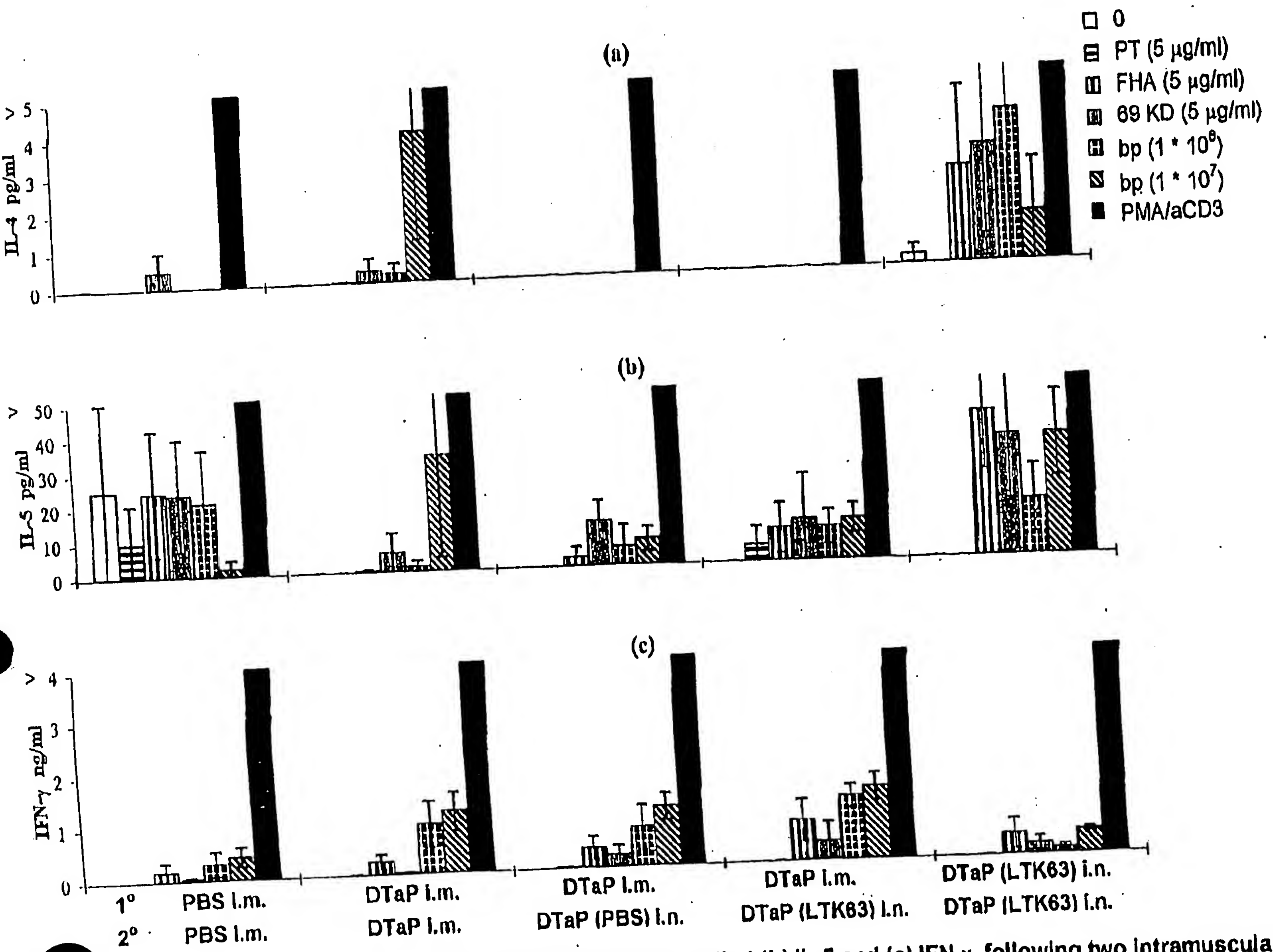
7



Fi[illegible] Spleen cell proliferation responses, to (a) pertussis antigens, (b) tetanus toxoid and (c) native CRM197 antigen, following two intramuscular, intranasal or int[illegible]uscular/intranasal immunizations with D[illegible]aP +/- LTK63.

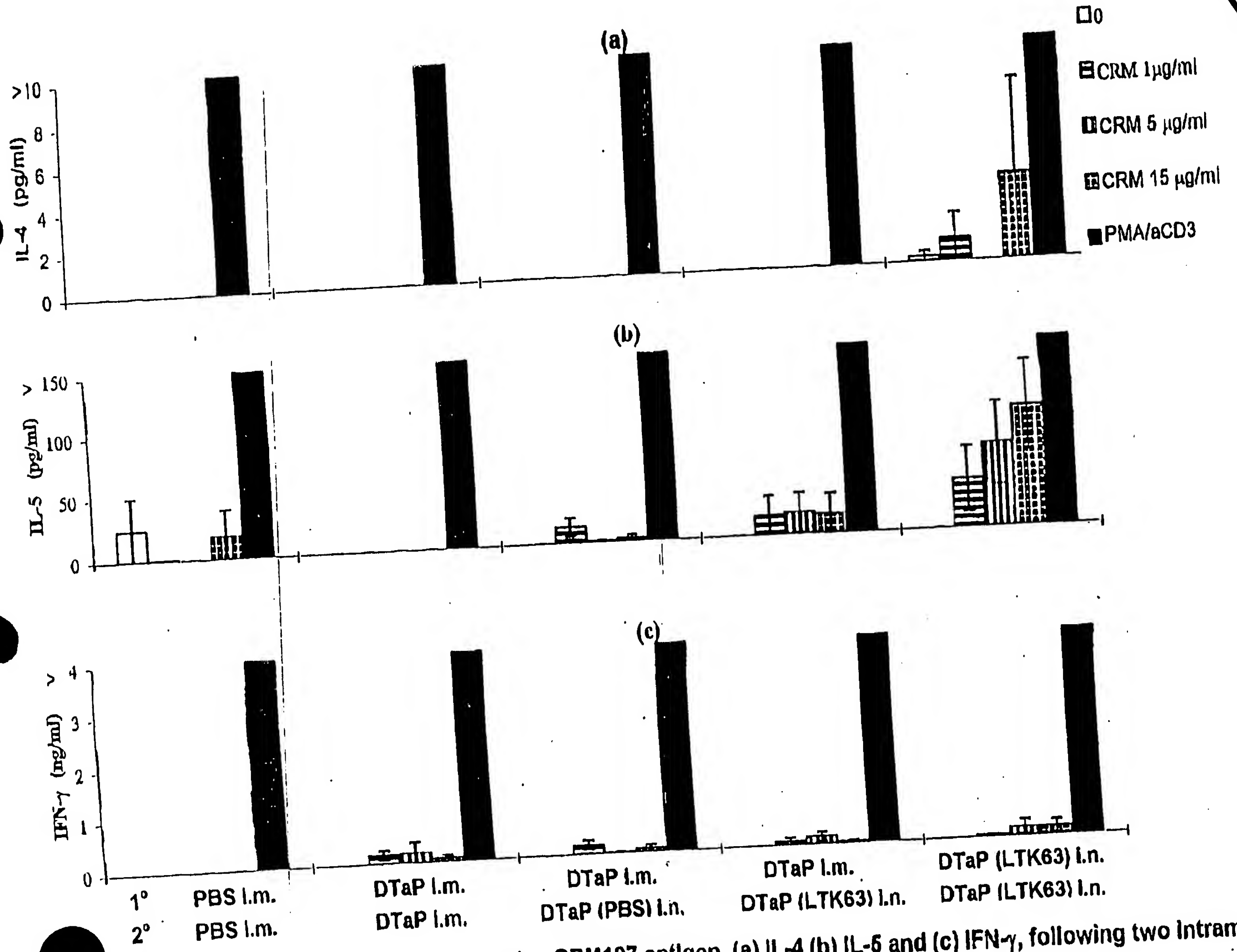
8

(S)



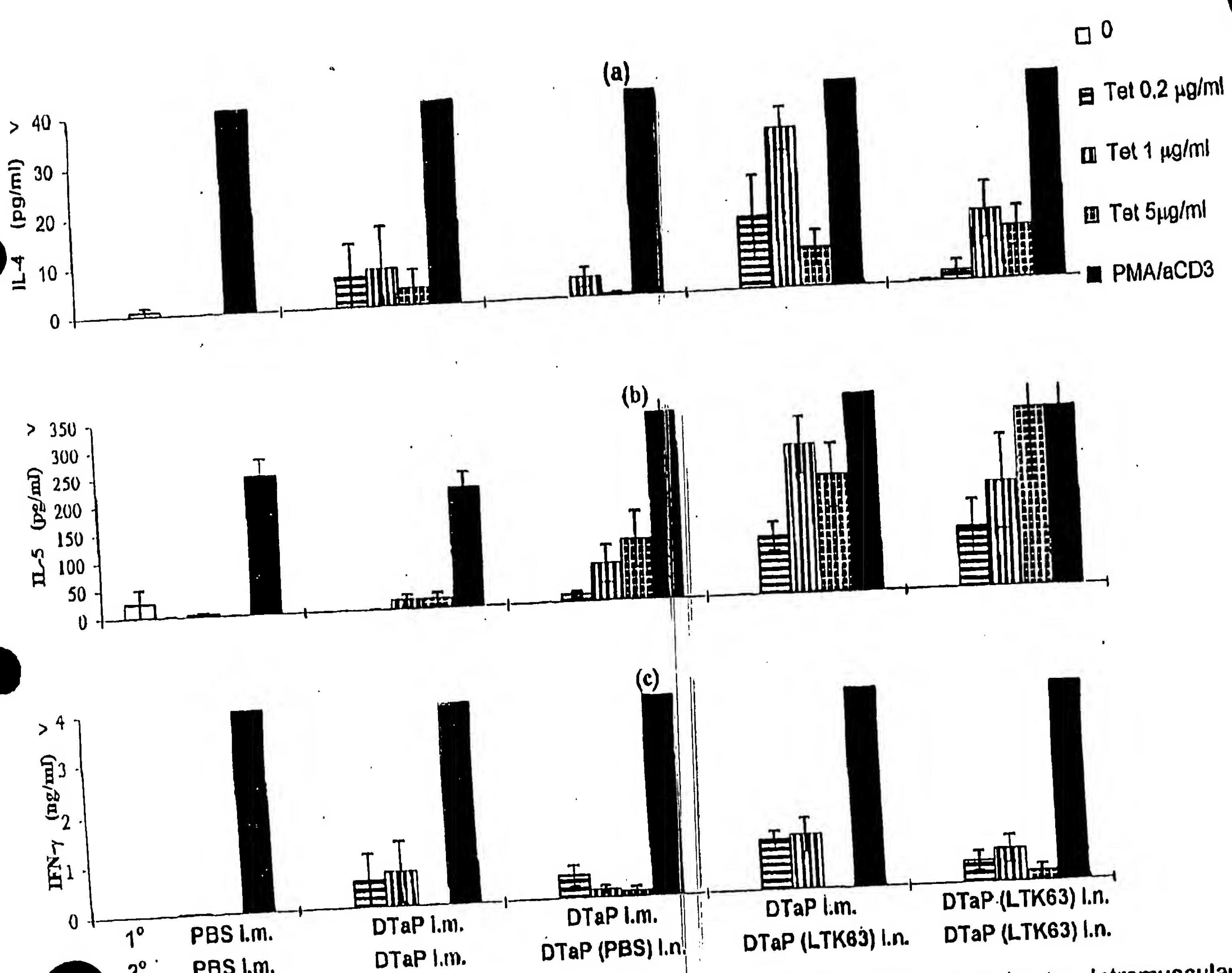
F[illegible] Spleen cell cytokine responses to pertussis antigens, (a) IL-4 (b) IL-5 and (c) IFN-γ, following two intramuscular, intranasal or intramuscular/intranasal immun[illegible]tions with DTaP +/- LTK63.

9

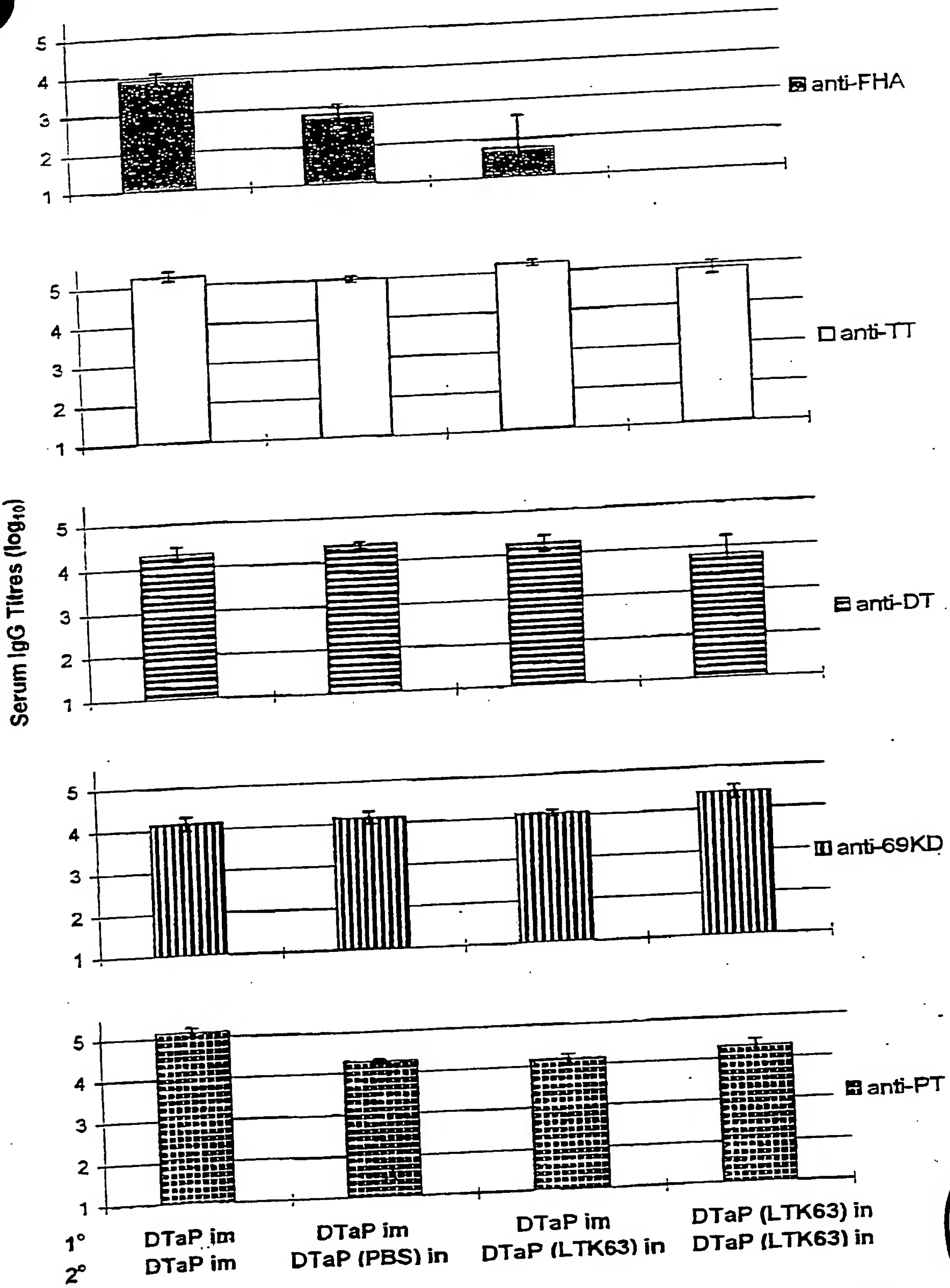


: Spleen cell cytokine responses to native CRM197 antigen, (a) IL-4 (b) IL-5 and (c) IFN-γ, following two intramuscular, intranasal or intramuscular/intranasal immu ations with DTaP +/- LTK63.

10

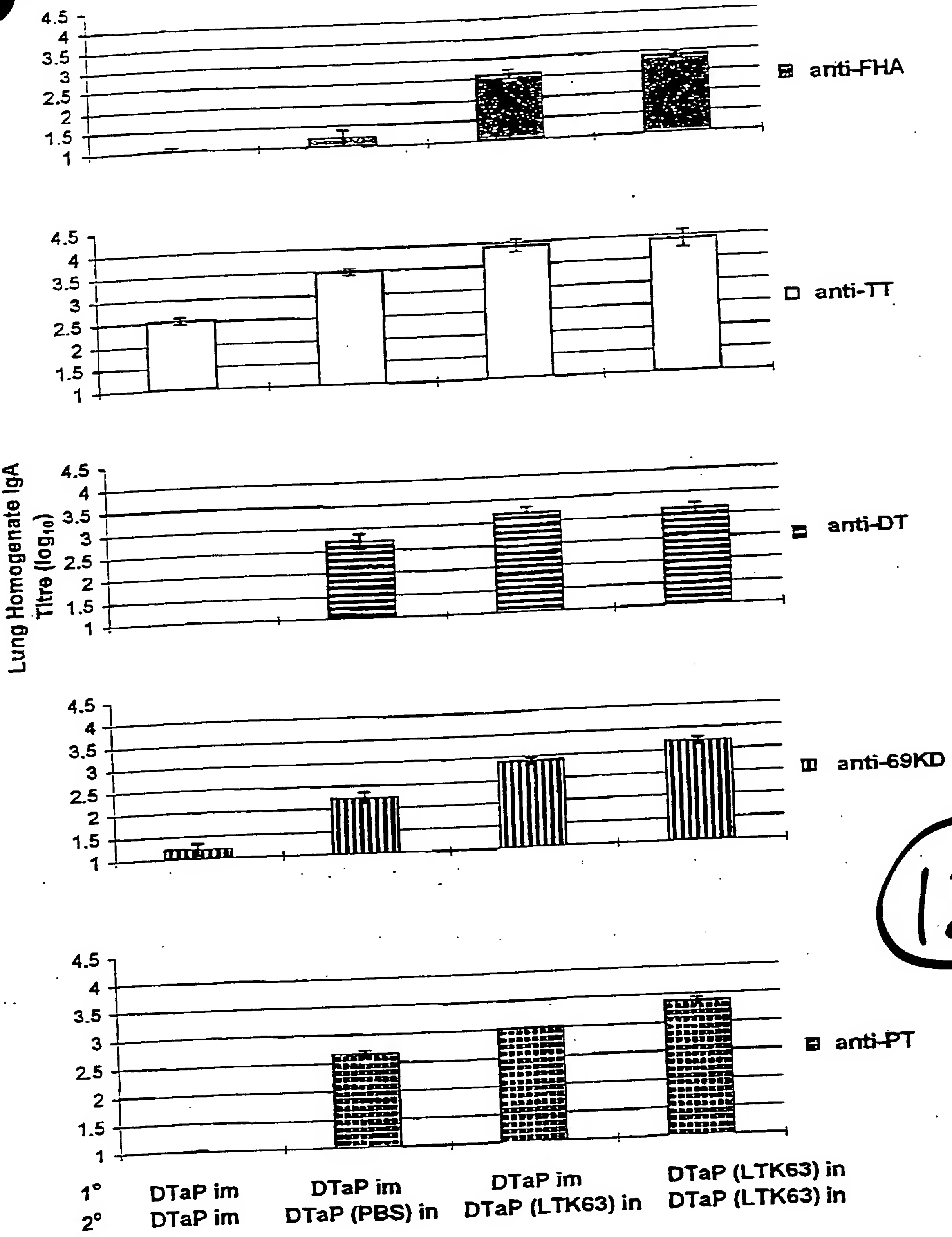


Fig[illegible]. Spleen cell cytokine responses to tetanus toxoid, (a) IL-4 (b) IL-5 and (c) IFN-γ, following two intramuscular, intranasal or intramuscular/intranasal immunizations wi[illegible] DTaP +/- LTK63.



⑪

Fig. 5: Serum IgG antibody titres following two intramuscular, intranasal or intramuscular/intranasal immunizations with DTaP +/- LTK63.

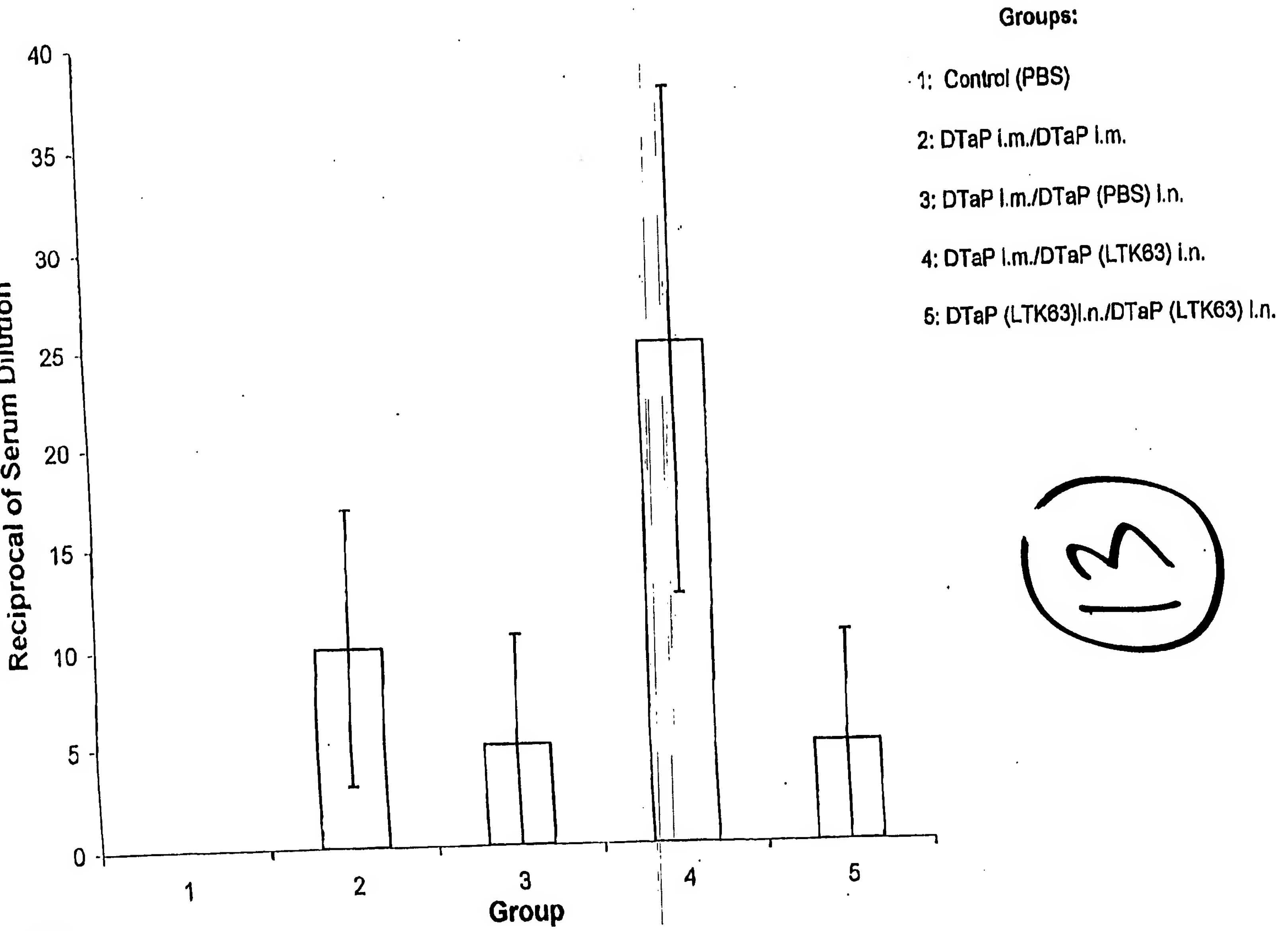


12

Fig. 6: Lung Homogenate IgA antibody titres following two intramuscular, intranasal or intramuscular/intranasal immunizations with DTaP +/- LTK63.

12

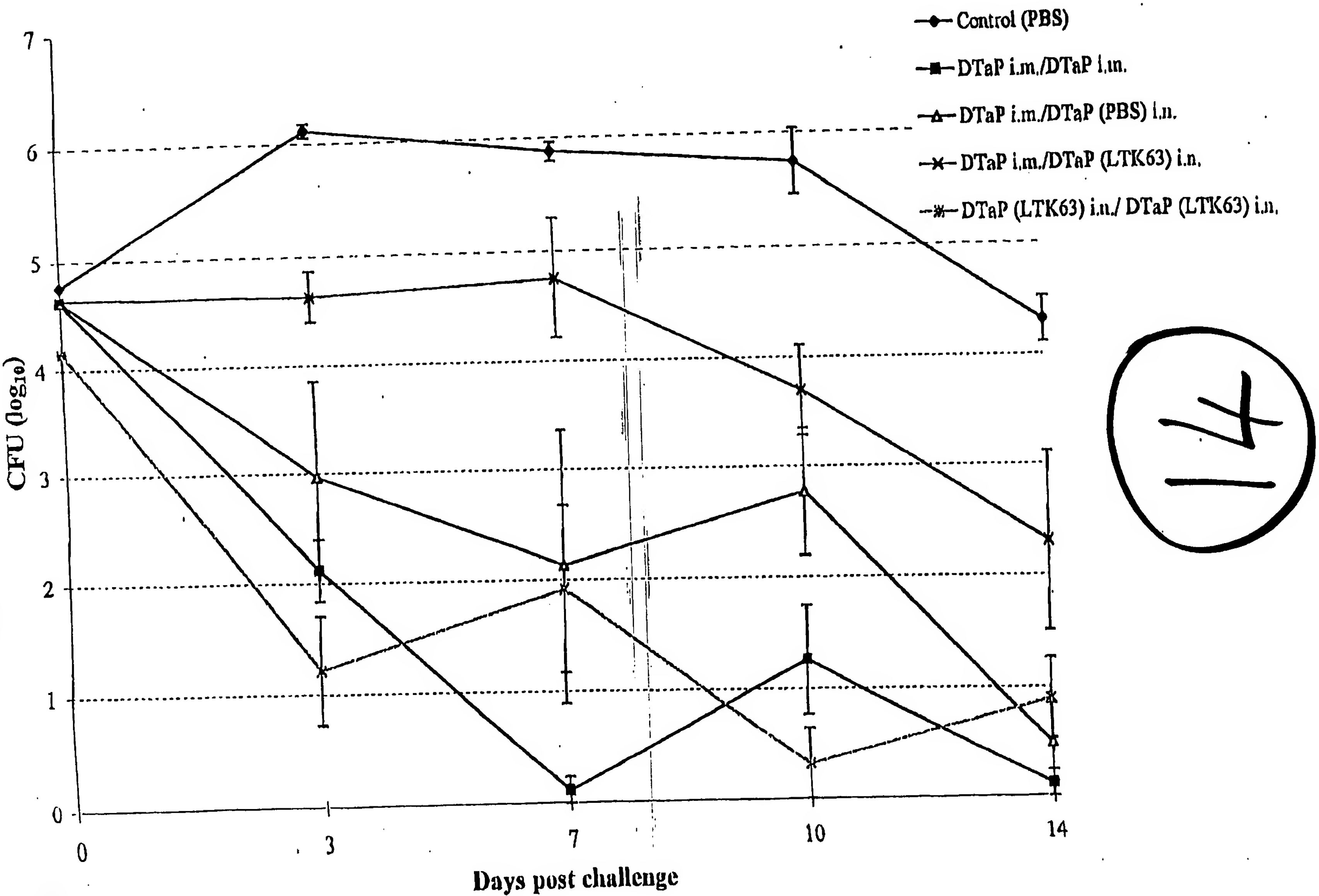
(13)



(13)

F[illegible] Serum anti-DT neutralizing antibody titres following two intramuscular, intranasal or intramuscular/intranasal immunizations with DTaP +/- LTK63.

(4)



14

Fig. 8: Bacterial clearance from the lungs of imm[illegible]ized mice following an aerosol challenge wit[illegible]ordetalla pertussis* bacteria.